Trends in Hematology/Oncology

haematologica 2002; 87:1324-1341

http://www.haematologica.org/2002_12/1324.htm

DNA methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias

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Background and Objectives. Methylation of DNA is a common epigenetic modification that plays an important role in the control of gene expression in mammalian cells. This process involves CpG dinucleotide sequences and is catalyzed by DNA-methyltransferase enzymes. Under physiological conditions, methylated CpG sites are only present in DNA sequences typical of bulk chromatin, where the DNA is inaccessible to transcription factors. In contrast, CpG islands of promoter regions are usually unmethylated (with few exceptions such as the genes on the inactive X-chromosome). DNA methylation abnormalities have recently emerged as the most frequent molecular changes in hematopoietic neoplasms.

Information Sources. The authors of the present review are currently working in the field of myelodysplastic syndromes and secondary leukemias and have contributed original papers to peer-reviewed journals. The material analyzed in the present review includes articles and reviews published in journals covered by the Science Citation Index, and abstracts presented at recent international oncology and hematology meetings.

State of the Art. Methylation and transcriptional status are inversely correlated, the hypermethylation of genes involved in cell-cycle control and apoptosis could have a pathogenetic role in the development of cancer. In particular, high-risk myelodysplastic syndromes (MDS) and secondary leukemias (SL) show a high prevalence of tumor-suppressor gene hypermethylation. The use of irreversible DNA methyltransferase inhibitors, such as 5-azacytidine and decitabine, appears to be a promising therapeutic option for the treatment of MDS and SL. Large clinical trials are still ongoing, but preliminary data recently published indicate for the first time that the natural history of MDS may be changed by a non-intensive treatment.

Conclusions and Perspectives. Treatment with demethylating agents, 5-azacytidine and decitabine, at present results in significantly higher response rates, improved quality of life, reduced risk of leukemic transformation, and improved survival, when compared to supportive care. Azacytidine and decitabine provide a new treatment option, and should be the treatment choice for elderly patients with high risk MDS. It is whorty in fact that azacytidine and decitabine are especially active in patients with poor prognosis MDS. The combination with histone deacetylase inhibitors may increase the efficacy of hypomethylating agents *in vivo*.

Key words: demethylation, 5-azacytidine, decitabine, transcription modulation, histone acetylation.

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As long suspected by many biologists, cancer appears to be a process triggered by genetic changes (e.g. DNA mutations) as well as epigenetic changes. Epigenetic changes include heritable traits that are mediated by changes in DNA other than nucleotide sequences.

Among epigenetic modulations, DNA methylation plays an important role in tissue- and stage-specific gene regulation,[1,2] genomic imprinting,[3,4] and X-chromosome inactivation[5] and has been shown to be essential for normal mammalian development.[6] This process has been shown to increase with age,[7] and during *in vitro* cell culture.[8]

Recent studies have demonstrated that both global DNA hypomethylation and regional hypermethylation occur during tumorigenesis.[9-11] Such aberrant DNA methylation occurs in a non-random, tumor-type specific manner. The eukaryotic genome is not uniformly methylated, but contains methylated regions interspersed with unmethylated domains.[12] During the process of evolution, the dinucleotide CpG has been progressively eliminated from the genome of higher eukaryotes and is present at only 5-10% of its predicted frequency.[13,14] Cytosine methylation appears to have played a major role in this process, because most of the disappeared CpG sites represent the conversion through spontaneous deamination of methylcytosine (also termed an *intrinsic mutagen*) to uracil and thus thymine. 5-methylcytosine differs from cytosine by the presence of a methyl group at posi-

tion 5 of the pyrimidine ring (Figure 1). The DNA methyltransferases methylate the cytosine residues in newly synthesized DNA, thus maintaining the parental pattern. Approximately 70% to 80% of the remaining CpG sites contain methylated cytosines in the genome of most vertebrates, including humans.[13,14] These methylated regions are typical of the bulk chromatin that represents the late replicating DNA, characterized by attendant histone composition and nucleosomal configuration, which makes it relatively inaccessible to transcription factors.[15] In contrast to the rest of the genome, smaller regions of DNA, called CpG islands, ranging from 0.5 to 5 kb, and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60% to 70%), with a CpG to GpC ratio of at least 0.6, and a normal dinucleotide CpG frequency.[16-18] Chromatin containing CpG islands is generally heavily acetylated, lacks histone H1, and includes a nucleosome-free region.[15] CpG islands localized in the promoter regions are usually unmethylated in normal tissues, regardless of the transcriptional activity of the gene. The main exceptions include untranscribed genes on the inactive X-chromosome and imprinted autosomal genes where one of the parental alleles may be methylated.[19] Tissue-specific genes without CpG islands are variably methylated, often in a tissue-specific pattern.[20,21]

DNA methylation is believed to contribute to cancer initiation and progression by gene expression inactivation.[22,23] This can have important consequences if the inactivated genes are essential for the control of normal cell growth, differentiation, or apoptosis. The mechanisms that regulate normal and aberrant methylation are not fully understood, nor are the mechanisms by which methylation interferes with transcription. These processes involve complex interactions between methyltransferases (DNMTs), methyl-CpG binding proteins (MBDs) and probably demethylase enzyme, as well as histone acetylases and deacetylases (HDAC), transcription factors, and chromatin structure.[24-26] The association of the methylation machinery, DNMTs and MBDs, with HDACs, provides a co-operative linkage in transcriptional silencing between DNA methylation and histone deacetylation.

Numerous examples of promoter hypermethylation of tumor suppressor genes, resulting in gene silencing and presumably conferring a growth advantage to involved cells, have been described and are summarized in Table 1.[27-29] A strong correlation between DNA hypermethylation, transcriptional silencing and tightly compacted chromatin has been established in many different systems.[2]

Work over the past 5 years has led to a remarkable increase in the knowledge of the mechanisms of chromatin structure modulation and has

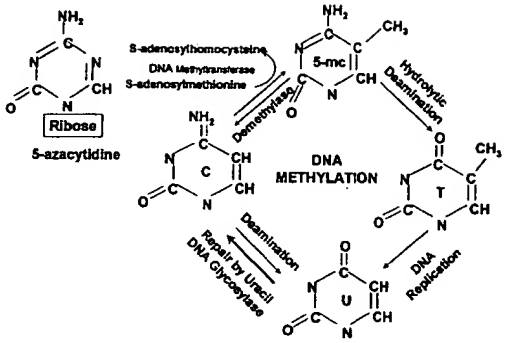

Figure 1. 5-methylcytosine differs from cytosine by the presence of a methyl group at position 5 of the pyrimidine ring. The DNA methyltransferase methylates the cytosine residues in newly synthesized DNA, thus maintaining the parental pattern. Unlike cytosine, 5-methyl cytosine is relatively unstable and its spontaneous deamination leads to uracil, making it an *intrinsic mutagen*.

Table 1. Selected genes hypermethylated in cancer.

Gene	*Function*	*Tumor types*
VHL	Promotes angiogenesis	Renal clear cell
p16/INK4A	Cyclin-dependent kinase inhibitor	Most solid, lymphoma
E-cadherin	Homotypic epithelial cell–cell adhesion	Ductal breast, thyroid, gastric, others
hMLH1	DNA-mismatch repair	Colon, gastric, and endometrial (MIN+)
BRCA1	DNA-damage repair	Breast (especially mucinous, medullary), ovarian
LKB1	Serine, threonine kinase	Hamartomatous colon, papillary, breast (Peutz-Jegher)
p15/^{INK4B}	Cyclin-dependent kinase inhibitor	Acute myeloid, lymphoid leukemias
ER	Receptor for estrogen-induced transcriptional activation	Breast, colon, leukemias, others
O6-MGMT	Repair of DNA guanosine methyl adducts	Brain, colon, lung, lymphoma
GSTPI	Prevention of oxidative DNA damage	Breast, prostate, renal
TIMP3	Inhibitor of tissue metallo-proteases	Colon, renal, brain
DAPK1	Kinase mediator of interferon-induced apoptosis	B-cell lymphomas, lung
p73	p53-like gene	Lymphomas

Based on data from Refs. #27-30.

revealed that chromatin is a dynamic structure which plays an important role in transcriptional regulation.[30] At least a major portion of chromatin remodeling appears to be accomplished through acetylation and deacetylation of the histone octamer *tails*. Many of the acetylation and deacetylation enzymes turn out to be known transcriptional enhancer and repressor proteins, respectively.[30] Heavily methylated, inactive regions of DNA (bulk chromatin) were also found to be enriched in hypoacetylated histones.[31] The process of DNA methylation, mediated by Dnmt1, may depend on or generate an altered chromatin state via histone deacetylase activity.[32] A major advance in understanding the link between DNA methylation, histone hypoacetylation, and gene silencing came from the recent studies of two laboratories. Both groups demonstrated that the methyl-binding protein MeCP2, known to be involved in transcriptional repression of methylated DNA, recruited a histone deacetylase, HDAC1, via the bridging protein Sin3a.[33-35] Recently, *in vitro* studies have also shown that DNMT1 physically interacts with either HDAC1 or HDAC2.[36,37]

The connection between DNA methylation and histone deacetylation could have therapeutic implications. DNA methylation and histone deacetylation appear to act as synergistic layers, although dense CpG island methylation is dominant for the maintenance of a silent state of genes during oncogenesis.[38] Inhibitors of histone deacetylases, alone or in combination with DNA hypomethylating agents, may be useful in reactivating tumor suppressor genes in cancer cells. A study using 5-azacytidine followed by trichostatin A (TSA), a potent and reversible HDAC inhibitor of human colon cancer and leukemia cells, robustly reactivated multiple hypermethylated genes such as *MLH1, TIMP3, p15^{INK4B}* and *p16^{INK4A}*.[38]

Methylation in hematologic malignancies

In hematologic malignancies, aberrant DNA hypermethylation is thought to be relevant for leukemogenesis.[39] For instance, during the progression of chronic myelogenous leukemia (CML), the *ABL1* promoter of the *BCR-ABL* fusion gene becomes significantly hypermethylated,[40,41] although this is not confirmed when using highly sensitive methylation assays.[42]

Hypermethylation of the calcitonin gene has been found in 65% of myelodysplastic syndromes (MDS),[43] in 95% of acute leukemias,[44] and is associated with an unfavorable clinical outcome in acute lymphoblastic leukemia (ALL).[45] Furthermore, aberrant hypermethylation and the consequent inactivation of the *p15^{INK4B}* tumor suppressor gene, is found in at least half of patients with CML,[42] ALL and acute myelogenous leukemia (AML),[45,46] and is associated with disease progression in MDS.[47,48] Moreover, hypermethylation of *p21$^{Cip1/Waf1}$* promoter, another cyclin-dependent kinase inhibitor belonging to the CIP/KIP family, has been found in about 40% of patients with ALL, and predicts a poor prognosis.[49]

In addition to these tumor-related genes, a number of other genes are concurrently hypermethylated in AML,[50] suggesting that there might be a more general dysregulation in the normal DNA methylation mechanisms, predisposing multiple genes to hypermethylation. Estrogen receptor methylation (ERM) is frequent in adult AML:[51] 61% of 261 patients had ERM values over 15% and were considered ERM$^+$. ERM decreases with increasing age (p=0.0001) and its levels are significantly lower in patients with French-American-British subtypes M4 or M5 AML (p=0.0019). Whereas ERM is not associated with a reduced complete remission rate after induction therapy, ERM$^+$ patients had significantly better overall and relapse-free survival.

When the methylation pattern of the CpG islands of the *calcitonin, estrogen receptor, E-cadherin, p15, p16, Rb, GST-Pi,* and *HIC1* genes was investigated in the bone marrow from 9 controls and 20 AML patients, all the control samples were essentially unmethylated for all the eight tumor-related genes studied. In contrast, 19 of 20 (95%) AML samples had an abnormal methylation pattern in at least one gene, and 15 of 20 (75%) had abnormal methylation patterns in two or more of the target genes.[50] Concurrent hypermethylation was further confirmed by some studies providing assessment of overall methylation changes by means of restriction landmark genomic scanning (RLGS). In contrast to techniques that allow assessment of the methylation status of individual genes, RLGS is a tool for the identification of genome-wide methylation changes in CpG islands, regardless of whether the genomic sequence is known. Using this technique, a preferential methylation of chromosome 11 was found.[52]

Methylation profiling[53] including estrogen receptor *(ER), p16, MDR1,CACNA1G, MINT1, MINT2, MDR1, THBS1,* and *PTC1MYOD1, PITX2,GPR37, SDC4,* performed in 36 cases of AML, demonstrated frequent hypermethylation of *ER* (47%), *MYOD1, PITX2, GPR37,* and *SDC4* in AML (47% to 64% of patients). In that study, *p15, p16, CACNA1G, MINT1, MINT2, MDR1, THBS1* and *PTC1* (2 promoters) methylation

Table 2. Hypermethylation at selected gene loci in MDS and acute leukemia.

Gene	Chromosome	Function	MDS (%)	AML (%)	ALL (%)
ER-α	6q25	Estrogen receptor	N.K.	70-90	90
E-Cadherin	16q22	Ca^2 dependent intracellular adhesion	N.K.	32-78	53
CALC1	11p15	Ca^2 bone resorbtion	25-60	50-90	45-90
HIC-1	17p13	Putative tumor suppressor gene		10	50-100
GPR37	7q31	G-protein-coupled receptor		47	
MDR	7q21	Drug efflux		31	
MINT1	5q13	CpG island hypermethylated in cancer		16	
MINT2	2p22	CpG island hypermethylated in cancer		8	
MyoD	11p15	Muscle-specific transcription factor		61	
p15	9p21	Cyclin-dependent kinase inhibitor	0-59	30-90	80
p16	9p21	Cyclin-dependent kinase inhibitor	0	0	0-40
PITX2	4q25	Homeotic gene		64	
PTC-A	9q22	WNT signaling		17	
PTC-B	9q22	WNT signaling		11	
SDC4	20q12	Surface heparan sulfate proteoglycan		56	
THBS1	15q15	Angiogenesis inhibitor		25	

Based on data from Refs. #39-51, and 102.

was relatively infrequent (6% to 31% of patients). For each of these CpG islands, the methylation density positively correlated to ERM density (rank order correlation coefficients, 0.32-0.59; 2-tailed $p <$ 0.058 for each gene). *MLH1* was unmethylated in all cases. In addition, *p15*, *MDR1*, and *SDC4* hypermethylation correlated to reduced levels of mRNA expression. There was an inverse correlation between age and the number of methylated genes. Thus, in a subset of AML cases, a *methylator phenotype* could be hypothesized (Table 2).[50-53]

Methylation depends on several functional DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B.[54] Mizuno *et al.* described a 5.3-, 4.4, and 11.7-fold increase in DNMT1, 3A, and 3B, respectively, in AML when compared to in normal bone marrow.[55] Although CML cells in chronic phase did not show significant changes, cells in blastic transformation showed 3.2-, 4.5-, and 3.4-fold increases in the levels of DNMT1, 3A, and 3B, respectively. Using methylation-specific PCR, it was observed that the $p15^{INK4B}$ gene was methylated in 24 of 33 (72%) AML cases. Furthermore, AML cells with methylated $p15^{INK4B}$ tended to express higher levels of DNMT1 and 3B. Thus, DNMTs were substantially overexpressed in leukemia cells in a leukemia type- and stage-specific manner and may contribute to the pathogenesis of leukemia by inducing aberrant regional hypermethylation.[55] An elegant study by Di Croce *et al.* recently established, for the first time, a mechanistic link between genetic and epigenetic changes in leukomogenesis.[56] The authors demonstrated that in acute promyelocytic leukemia (APL) the leukemia-promoting PML-RARA fusion protein induces gene hypermethylation and silencing by recruiting DNMT1 and DNMT3A to the promoter of its target gene *RARβ2*. A previous study showed that an additional oncogenic transcription factor induces aberrant hypermethylation of target gene promoters. The mechanism of transcription repression exerted by PML-RARA was the recruitment of HDAC complex.[57] Consistent with this finding, Di Croce *et al.* showed that PML-RARA-induced repression of *RARβ2* was only partially relieved by either 5-aza-2'-deoxycytidine or TSA treatment of APL cells.[56] Notably, only simultaneous treatment with 5-aza-2'-deoxycytidine and TSA completely restored *RARβ2* gene expression. Thus, all these observations, taken together, suggest the intriguing scenario in which the newly methylated CpGs become docking sites for methyl-binding proteins, which in turn interact with both HDAC complexes and DNMTs, finally leading to the spreading of hypermethylation to the neighboring DNA regions.

Clinical and laboratory features of myelodysplastic syndromes and role of hypermethylation in their progression to secondary leukemia

Myelodysplastic syndromes (MDS) include a heterogeneous group of clonal myeloid stem cell disorders characterized by peripheral cytopenias and dysplasia of bone marrow progenitor cells.[58] A normo- to hypercellular bone marrow contrasts with peripheral cytopenia, anemia, thrombocytopenia, and/or neutropenia, as signs of ineffective hematopoiesis. Approximately 10% of the patients present with hypocellular marrow.[59] The natural history of these diseases ranges from a chronic course that may span years to a rapid course towards leukemic progression. Unfortunately, the nomenclature and classification systems used to describe these conditions are cumbersome and contentious. In 1982, the French-American-British (FAB) group proposed a classification based on morphologic features of blood and bone marrow, namely bone marrow and peripheral blood blast cell count, ringed sideroblasts, number of monocytes in peripheral blood, and Auer rods.[58] Five subgroups with significantly different prognoses were established: refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of

blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML) (Table 3). This classification served as the standard for the evaluation of MDS for nearly 2 decades, though further characteristics of MDS subtypes—for example, hypoplastic MDS[59] and MDS with bone marrow fibrosis—were not considered by the FAB classification.[60]

In addition, some studies provided evidence that patients with CMML and white blood cell (WBC) counts above 13000/μL had hematologic and clinical characteristics of myeloproliferative disorder.[61-63] Other studies showed that the entities defined by the FAB group were heterogeneous, not only in terms of prognosis but also of morphologic features.[60,64-67] In 1999, the *World Health Organization* (WHO) published a revised classification of MDS[68] (Table 3). The definitions of RA and RARS became more precise and included the presence of dysplastic features in the erythroid lineage only. At present, the definition of RAEB remains unchanged, though some investigators suggest further discrimination between RAEB I with 5% to 10% blasts and RAEB II with 11% to 20% blasts in the bone marrow. Three new subgroups were incorporated: (1) refractory cytopenia with multilineage dysplasia (RCMD), which is equivalent to RA or RARS in the FAB classification in the presence of dysplastic features of 2 or 3 cell lineages; (2) del (5q) syndrome, characterized by dysplastic changes in the erythroid lineage only, thrombocytosis, and hypolobulated micromegakaryocytic hyperplasia (an isolated interstitial deletion of the long arm of chromosome 5 is now a distinct entity); and (3) MDS unclassifiable (MDS unclass). Two other FAB subgroups have been excluded from the new MDS classification: RAEB-T, because of some similarities in biologic features and treatment strategies with acute myeloid leukemia (AML), and CMML, because of its close relation to myeloproliferative diseases. CMML, together with atypical chronic myeloid leukemia (aCML) and the juvenile form of CMML, JMML, is now part of a new disease group called myelodysplastic/myeloproliferative diseases, and RAEB-T ceases to exist because of the new threshold of 20% blasts in the bone marrow set for AML. The advantages and disadvantages of the WHO classification are still a subject of lively, controversial discussion.[69]

Myelodysplastic syndromes can arise *de novo* or be related to exposure to ionizing radiation or myelotoxic drugs.[70-74] The crude incidence of *de novo* MDS is about 3-4 new cases per 100.000 persons per year, although some authors have reported a higher incidence.[71,72,74] The incidence rate increases steeply and constantly with age.[72] This increase is markedly higher in females than in males, and is likely to be due to the higher proportion of females in the elderly population.[71] The incidence of therapy-related MDS (t-MDS) is yet to be defined in larger series of patients. In the M.D. Anderson series before 1991, t-MDS represented 27% of all MDS (73), and were probably more common than overt therapy-related AML (t-AML), considering that macrocytosis and cytopenias, the hallmarks of MDS, can be overlooked in patients having been treated for a previous neoplasm.

MDS is a well recognized acquired genetic disorder with characteristic cytogenetic abnormalities found in 50–60% of the patients.[75-77] Cytogenetic abnormalities are similar in both *de novo* and t-MDS, but are more frequent in t-MDS. Deletion or loss of chromosomes 5 and 7 is commonly associated with exposure to alkylating agents.

A clonal evolution associated with progressive bone marrow failure and transformation into AML occurs in 10–60% of cases depending on the MDS subtype and the cytogenetic pattern.[70-76] According to the data of the co-operative study reported by Mufti,[70] progression into overt leukemia is very frequent in RAEB-t (60%) and in RAEB (44%) and rare in RA (12%) and RARS (8%).

Various prognostic scores have been developed to predict patients' prognosis more accurately. The

Table 3. FAB and WHO Classification of MDS.

FAB subtype	WHO subtype	BM blasts (%)	PB blasts (%)	Monocytes >1×10⁹/L in PB	Ringed sideroblasts >15%
RA	RAa	<5	<1	–	–
RARS	RARS[a]	<5	<1	–	+
	Refractory cytopenia with multilineage dysplasia[b]	<5	<1	–	+/–
	5q- syndrome	<5	<1	–	+/–
RAEB	RAEB	5-20	<5	–	+/–
	MDS unclassifiable	<1-20	<5	–	+/–
CMML	CMML<13 1×10⁹ WBC/L	<1-20	<1-20	+	+/–
RAEB-T	AML	21-30		+/–	+/–

AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; RA, refractory anemia; RAEB, refractory anemia with excess of blasts; RAEB-T, refractory anemia with excess of blasts, in transformation; RARS, refractory anemia with ringed sideroblasts. [a]Only erythroid lineage; [b]At least two lineages involved.

most widely accepted system, the *International Prognostic Scoring System* (IPSS) (Table 4), was introduced in 1997 by a group headed by Greenberg.[78] The IPSS defines four prognostic groups on the basis of three criteria: number of cytopenias, blast counts, and cytogenetics (Table 5). A great proportion of patients with t-MDS have a high *International Prognostic Score* and are characterized by poor prognosis: independently of a 50% progression rate to AML, patients with t-MDS have a poorer prognosis than patients with *de novo* MDS.[73,74] *p15* methylation appears an important step in MDS evolution.[48,79] The $p15^{INK4B}$ gene, and its functional homolog $p16^{INK4A}$ gene, encode proteins which negatively regulate the cell cycle by inhibiting the cyclin-dependent kinases 4 and 6, which control the progression of cells from G1 to S phase.[80] The expression of the cyclin-dependent kinase inhibitor (CDKI) $p15^{ink4B}$ (p15) is upregulated during *in vitro* granulocytic and megakaryocytic differentiation of normal $CD34^+$ hematopoietic progenitors.[81] Inactivation of the $p15^{INK4B}$ and $p16^{INK4A}$ genes by homozygous deletion has been detected in many cancers, suggesting that they are candidate tumour suppressor genes.[82,83] Among the hematologic malignancies, homozygous deletion of the $p16^{INK4A}/p15^{INK4B}$ gene is a frequent event in ALL and non-Hodgkin's lymphoma,[84-86] but rarely occurs in myeloid malignancies.[87,88] Hypermethylation is an alternative mode of *p15* inactivation. Aberrant methylation of CpG islands in the *p15* promoter region commonly occurs in MDS, such as RAEB or RAEB-T, and is associated with loss of *p15* expression.[48,89] *p15* hypermethylation was found in AML and MDS concomitantly with disease progression.[48,79,90] Patients with $p15^{IKN4B}$ methylation at diagnosis had a significantly shorter survival than those with a normal methylation pattern.[90] The incidence of $p15^{INK4B}$ gene methylation in *low-risk* (RA and RARS) or *high-risk* MDS (RAEB, RAEB-T and CMML), respectively, is constant during the course of the disease.[90] With the assay used in that work (methylation-sensitive polymerase chain reaction), *p15* methylation was not detectable throughout the course of *low-risk* MDS and ranged from 23% at diagnosis to 30% at advanced stages in *high-risk* MDS. Acquisition of $p15^{INK4B}$ methylation during follow-up accompanied disease progression.[48,79,90] $p15^{INK4B}$ methylation ranged from 60% to 75% in AML evolved from previous MDS. Patients with $p15^{INK4B}$ gene methylation at diagnosis or at subsequent follow-up had a significantly higher chance of disease progression to AML than those without gene methylation.[90] This suggests that $p15^{INK4B}$ gene methylation might play an important role not only in disease progression, but also in the early development of some high-risk MDS. Most patients with $p15^{INK4B}$ methylation, detected either at initial diagnosis or during the disease course, progress to AML, indicating *p15* methylation as a marker of leukemic transformation in MDS.[79,90] Methylation could also play a role in therapy-related leukemias. In patients with therapy-related leukemia, the frequent microsatellite instability could be related to a deficiency of genes involved in DNA mismatch repair (MSH2 and MLH1)[91-95] or to their silencing by methylation. We found MLH1 hypermethylation in patients with therapy-related leukemia, but not in patients with *de novo* leukemia (*unpublished observation*).

Table 4. MDS International Prognostic Scoring System (IPSS).

	Score				
	0	*0.5*	*1*	*1.5*	*2*
BM blasts (%)	<5	5-10	–	11-20	21-30
Karyotype	Good*	Intermediate#	Poor°		
Cytopenia° (number of lineages involved)	0/1	2/3			

*Cytopenia: hemoglobin < 10 g/dL, neutrophils < 1.5×10^9/L, platelets < 100×10^9/L; BM: bone marrow. *(normal, -Y, 5q-, 20q-); #(other abnormalities); °(complex,chromosome 7abnormalities).*

Table 5. Prognostic groups of MDS according to the IPSS.

Total score value	*Risk group*	*Median survival (years)*
0	Low	5.7
0.5-1	Intermediate 1	3.5
1.5-2	Intermediate 2	1.2
> 2.5	High	0.4

Rationale for the use of demethylating agents in MDS and secondary leukemia

Cytosine methylation is an epigenetic process that may be modulated by biochemical and biological manipulations. The inhibition of methyltransferase results in reactivation of genes silenced.[96] DNA hypomethylating agents are effective *in vitro* and *in vivo* in reducing hypermethylation present in patients affected by cancer, leukemia and myelodysplasia.[97,98] The demethylat-

ing agents (5-azacytidine and 5-aza-2'-deoxycytidine/decitabine) can restore the normal demethylated state of several types of tumor suppressor genes, including p16, E-cadherin, h MLH1, VHL, p15, increasing their expression *in vitro*[99-101] and *in vivo*.[102] Still, MDS CD34+ cells only poorly differentiate to the megakaryocytic lineage following demethylation with azacytidine.[103] Thus, it is possible that the profound proliferation, survival, and differentiation defect of the neoplastic clone cannot be completely overcome by this demethylating drug alone. *In vivo*, however, treatment of MDS patients with another demethylating drug, decitabine often results in an early and sometimes massive increase in platelet counts.[104] Considering that DNA methylation and histone deacetylation act as synergistic layers for the silencing of genes in cancer, the combination of demethylating agents with inhibitors of acetylation could be a logical approach to reactivate silenced genes.[38]

Chemical structure and mechanisms of action of demethylating agents

The cytidine analogs modified in position 5 of the pyrimidine ring, such as 5-azacytidine (azacytidine), 5-aza-2'deoxycytidine (decitabine), pseudoisocytidine, and 5'-fluoro-2'-deoxycytidine, are potent inhibitors of DNA methylation (Figure 2). Decitabine has the highest efficacy.[105] The hypomethylating effect of cytidine analogs appears to depend primarily on the presence of an altered C5 position; other cytidine analogs, such as ara-C, 6-azacytidine, and gemcitabine, do not possess this property. Arabinofuranosyl-5-azacytidine (fazarabine) showed a broad antitumor activity in experimental models, but not in clinical trials.[106] Azacytidine and decitabine, both synthesized by Sorm *et al.* in 1964[107,108] are characterized by a strong *in vitro* and *in vivo* antileukemic effect at high concentrations, while showing a differentiation-inducing activity at lower concentrations in cell line models[109-111] and primary myeloid leukemic blasts.[112] Thus, the efficacy of azacytidine and decitabine as antineoplastic agents appears to result from two distinct mechanisms: cytotoxicity (high dose), and induction of hypomethylation leading to cellular effects that are distinct from immediate cytotoxicity (low dose). Both compounds are activated to triphosphate (azacytidine by uridine-cytidine kinase, decitabine by deoxycytidine kinase) and are degraded by cytidine deaminase. Azacytidine, being a ribonucleoside, incorporates into RNA and, to a much lesser extent, into DNA. Decitabine only incorporates into DNA. Incorporation into RNA produces disassembly

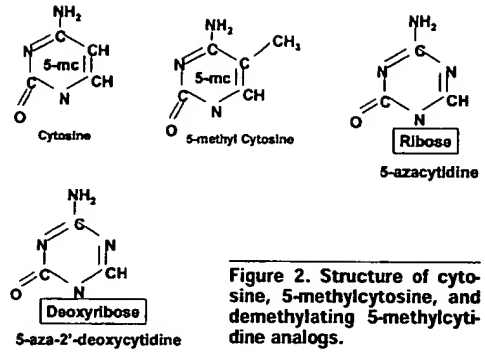

Figure 2. Structure of cytosine, 5-methylcytosine, and demethylating 5-methylcytidine analogs.

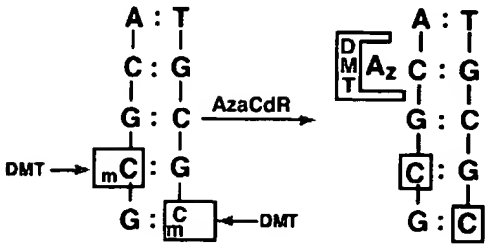

Figure 3. 5-aza-2'-deoxycitidine (Aza CdR, Az) inhibits DNA methyltransferase and induces hypomethylation and transcription of previously quiescent genes (modified from Silverman *et al.*, 2002).[123]

of polyribosomes, defective methylation and acceptor function of transfer RNA, and marked inhibition of protein production. Insertion into DNA results in covalent linking with DNA methyltransferases and blocking of DNA synthesis[113] that ultimately result in cytotoxicity.

Azacytidine is highly cytotoxic to cells in S phase and mainly exerts its action on rapidly dividing cells. Unlike ara-C and azacytidine, decitabine does not block cell-progression to S-phase, suggesting that its cytotoxic activity is not self limiting. This indicates that decitabine has a higher *in vivo* cytotoxicity than ara-C or azacytidine.[114]

Both azacytidine and decitabine may also exert antitumor activity through induction of DNA hypomethylation, by forming a covalent complex with the major DNA methyltransferase (now termed DNMT1). DNA methyltransferases catalyze the transfer of a methyl-group from the methyl-donor S-adenosyl-methionine into the 5' position in the cytosine ring (Figure 1).[1] DNMT1 is responsible for

duplicating the DNA methylation pattern during DNA replication.[115]

Azacytidine and decitabine effectively deplete the cell of functional DNA methylating activity, which results in profound hypomethylation after several rounds of DNA replication (Figure 3). DNMT1 is considered a *bona fide* anticancer target at different levels.[116] It induces DNA methylation, but in addition contains two domains that can recruit HDAC1[32] and HDAC2.[36] Drugs inhibiting histone deacetylation could further reduce DNMT1 activity.[38] Through inhibition of DNA methylation, azacitidine and decitabine, also at low doses, induce re-expression of silenced gene.[96] Decitabine, at concentrations inhibiting DNA methylation, is 30-fold more active than azacytidine.[117] The combination of HDAC inhibitors with azacytidine/decitabine is synergistic in reactivation of silenced genes (Figure 4).

Clinical studies with azacytidine and decitabine in myelodysplastic syndromes and secondary leukemias

Preliminary studies, initiated many years ago (most of them designed before the discovery of the demethylating activity and using azacytidine mostly in the USA, decitabine in Canada and Europe) showed promising results in high-risk MDS and resistant or relapsed leukemias.[118] In other studies published after 1984 by the Cancer And Leukemia Group B (CALGB), low-dose azacytidine showed efficacy in about 50% of MDS patients.[119] In a single-arm phase I/II trial, enrolling patients with mostly RAEB and RAEB-T, using monthly azacytidine, 75 mg/m²/day continuous infusion for 7 days, 49% of patients responded, with 37% having trilineage responses, either complete or partial. Complete remission was achieved by 5 of 43 patients and partial remission by 11 patients. Patients who did not respond after 4 months were taken out of the study. The median survival for all patients was 13.3 months, while the median duration of remission was 14.7 months (Table 6). Interestingly, the best response was observed after a mean of 3.8 treatment courses (range 2-11 courses), indicating that repeated applications of azacytidine probably are necessary to achieve maximum efficacy. The most frequent side effects were nausea and/or vomiting (63%), followed by diarrhea (30%).[119]

A subsequent study (*CALGB 8921 trial*), using subcutaneous bolus injection of azacytidine with the identical total dose of 525 mg/m² per course, also produced a response in about 50% of patients, with 27% having trilineage responses (Table 6).[120] A mild activity, without significant myelosuppression, was demonstrated in MDS patients using an even lower dose of azacytidine (15 mg/m²/day for 14 days i.e. 210 mg/m² total dose).[121]

Between 1991 and 1998, Rugo *et al.* treated 92 patients, mostly with high-risk MDS or secondary AML, with the outpatient schedule pioneered by Silverman *et al.* of 75mg/m²/day subcutaneously for 7 days (repeated every 28 days for six cycles) and reported 61% overall responses (13% and 19% CR and PR, respectively) in a retrospective analysis of this compassionate-use program.[122] Two patients

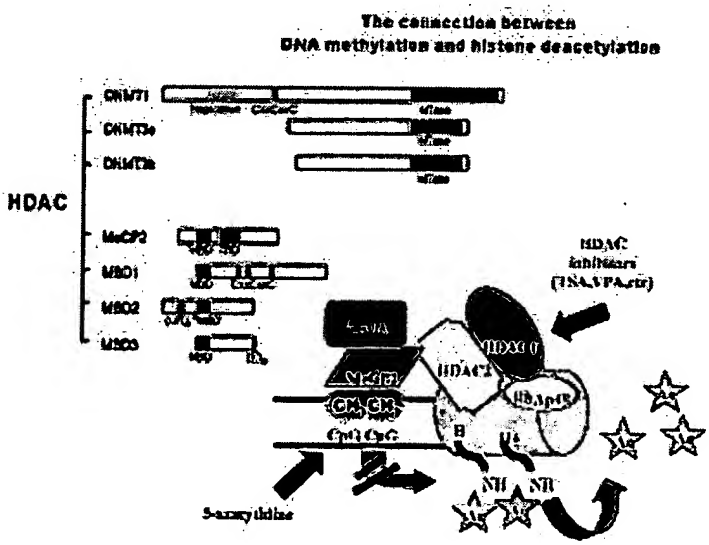

Figure 4. The combination of HDAC inhibitors with azacytidine/decitabine is synergistic in reactivation of silenced genes *(courtesy of Dr. Clara Nervi).*

Table 6. CALBG 8421, 8921 trials: response to 5-azacytidine in MDS.[119,120]

	Trial 8421 Intravenous n (%)	Trial 8921 Subcutaneous n (%)
Patients enrolled	49	70
Patients with evaluable data	43	68
Complete response	5 (12)	8 (12)
Partial response	11 (25)	10 (15)
Improvement	5 (12)	18 (27)
Total response	21 (49)	36 (53)

Table 7. CALGB 9221 trials: azacytidine versus supportive care.[123]

	Supportive care	Azacytidine
Patients evaluated, n	92	99
Complete response, n (%)	0 (0)	7 (7)*
Partial response, n (%)	0 (0)	16 (16)*
Improved, n (%)	5 (5)	37 (37)*
Total, n (%)	5 (5)	60 (60)*

*$p = 0.01$; *$p < 0.0001$.

with complete hematologic response also had a cytogenetic remission. The major toxicity in this study was mild to moderate nausea and vomiting. These promising results led to a phase III CALGB trial including 191 patients in three arms: subcutaneous azacytidine (n=99), versus standard supportive care (n=92), with cross-over to azacytidine treatment after 4 months (and in some instance, 2 months) in case of disease progression (n=46). The trial design (*CALBG 9221*) is illustrated in Figure 5. Significant differences were reported in complete remission, partial remission rate, and *improved* categories between the azacytidine-treated group and the group receiving supportive care only (Table 7).[123] The treatment-related mortality was low (death of only one patient on the treatment arm was attributable to the drug treatment) and the median duration of response was 15 months. Highly significant differences were noted in the patients' times on-study before exiting the trial due to lack of response, transformation to leukemia, low platelet counts (<20,000/μL), or death. A statistical analysis of competing variables demonstrated a significant difference in time on supportive care of 22 months versus 12 months (p=0.0034). By intention to treat, transformation to AML was 2.8-fold more frequent in the supportive-care group than in the azacytidine group (p=0.003), suggesting that azacytidine can prevent transformation to acute leukemia. The overall survival in the cross-over arm was similar in patients crossing from the *supportive care* arm to azacytidine, when compared to patients receiving induction with azacytidine (18 months versus 14 months). A quality-of-life analysis showed that patients treated initially with azacytidine had a significant improve-

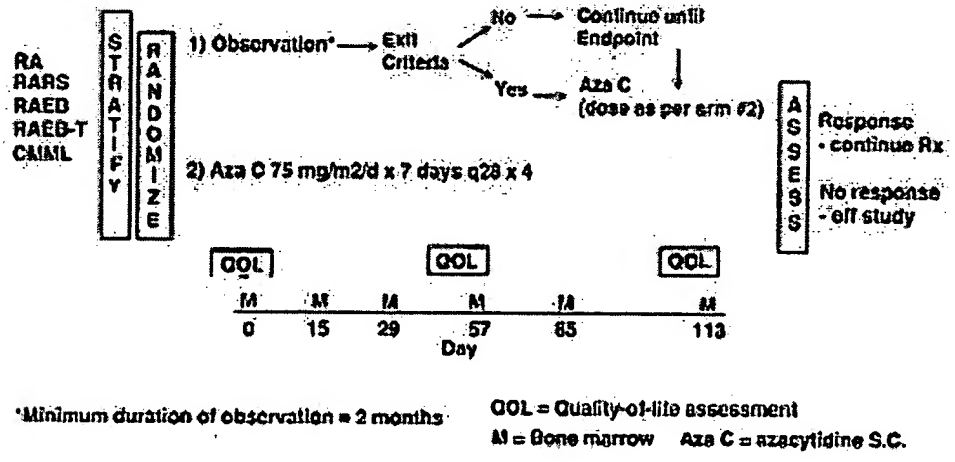

Figure 5. Trial design of the Cancer and Leukemia Group B (CALGB) 9221 study (*adapted from Silverman 2002*).[123]

ment over time in fatigue, dyspnea, physical functioning, and physiological distress, compared to those receiving supportive care only.[124] These results are summarized in Tables 6 and 7. In summary, this study showed, for the first time, that the natural history of MDS may be changed by a non-intensive drug treatment, with improved quality of life for patients treated with azacytidine C.[123-124]

Low-dose decitabine was first employed in the treatment of MDS and leukemia by the Aviano group in Italy.[125] Ten patients affected by advanced MDS, 2 with RAEB and 8 with RAEB-T were treated with decitabine at a daily dose of 45 mg/m^2, divided into three 4-hour infusions for 3 days (6 patients, total dose 135 mg/m^2) or of 50 mg/m^2 given as continuous infusion for 3 days (4 patients, 150 mg/m^2). Treatment with decitabine resulted in a significant increase in circulating neutrophils, platelets, and hemoglobin, when compared to pretreatment values, in over 50% of patients. These changes were accompanied by an improvement in the bone marrow myeloid *relative differentiation index* (median 5-fold increase in the whole group of patients) and of the myeloid-to-erythroid cell ratio (median 2-fold increase) in most of the patients. In 4 of 10 patients, a complete normalization of peripheral blood and bone marrow picture (complete hematologic response) was obtained. A slow but progressive reduction of early leukemic progenitors was observed in most of the patients. A transient slight bone marrow hypoplasia occurred in less than 50% of patients, while severe marrow aplasia was never observed. Extra-hematologic toxicity was mild in all patients.[125]

A considerable activity of high-dose decitabine was also demonstrated in leukemic patients.[126] A total of 12 patients with poor prognosis leukemia, mostly secondary to MDS, were treated with decitabine at a dose of 90-120 mg/m^2 as a four-hour intravenous infusion, three times daily for three consecutive days every four to six weeks (810 and 1080 mg/m^2 total dose, respectively).[126] A minimum of two courses were required for response evaluation and to consider a patient as not having had a therapeutic response. Ten patients were fully evaluable for response: 3 patients achieved complete remission (CR) and 1 patient a partial remission (PR). Extra-hematologic toxicity was generally mild. As for the mechanism of action, both a differentiation induction effect and, not unexpectedly, a cytotoxic mechanism have been observed. In particular, CRs and PRs were possibly also due to a differentiation effect of leukemia cells, as suggested by the slow kinetics of remission and by immunophenotyping studies.[126,127]

These data were confirmed with a low-dose schedule by Wijermans *et al.*[128] 5-aza-2'-deoxycytidine (decitabine) at low doses (120-225 mg/m^2 total dose), as a 72-h continuous infusion, was tested in a group of 29 elderly patients with high-risk MDS. In 15 patients (54%) a response was observed, with 8 CR, including patients with bad prognostic cytogenetics. The actuarial median survival from the start of therapy was 46 weeks. The only (and major) toxicity was myelosuppression, leading to a prolonged cytopenia and 5 toxic deaths (17%) in this high-risk patient group. It was concluded that decitabine is an effective drug in the treatment of MDS. Myelotoxicity is the major adverse effect. The same group confirmed these preliminary data in a multicenter study.[129] Between June 1996 and September 1997, 66 patients (median age, 68 years) from seven centers in the Netherlands, Belgium and Germany received decitabine 45 mg/m^2/day (administered as three 15 mg/m^2 infusions given over 4 h) for 3 days every 6 weeks (total dose 135 mg/m^2, as in the Aviano trial of MDS). For patients who achieved complete remission after two courses, two further cycles were administered as consolidation therapy. In the case of stable disease, improvement, or partial response, a maximum of six cycles was administered. The primary end-points were response rate and toxicity. The secondary end-points were response duration, survival from the start of therapy and overall survival.

The overall response rate was 49%, with a 64% response rate in patients with a high-risk International Prognostic Score (IPSS),[78] and a response duration of 39 and 36 weeks for patients who reached PR or CR, respectively (Table 8). The actuarial median survival time from diagnosis was 22 months and from the start of therapy was 15 months. For the IPSS high-risk group, the median survival was 14 months. The median progression-free survival was 25 weeks.

Myelosuppression was rather common, the treatment-related mortality rate was 7% and was primarily associated with pancytopenia and infections. Significant responses were observed with regard to megakaryopoiesis, with increases in platelet counts occurring after one cycle of decitabine therapy in the majority of responding patients. In summary, decitabine therapy was effective in half of the patients with high-risk MDS and was especially active in those with the worst prognoses. Myelosuppression was the only major adverse effect observed.

In a recent update, data were analyzed of 162

Table 8. Responses to decitabine among different IPSS risk groups of MDS.[129]

	IPSS Risk Group				
	Intermediate I	*Intermediate II*	*High risk*	*Total n*	*%*
Toxic death	2	1	2	5	8
Disease progression	2	10	5	17	26
Stable disease	8	2	2	12	18
Improvement	2	5	9	16	24
Partial response	1	1	1	3	4
Complete response	1	6	6	13	20
Total	16	25	25	66	100

uniformly decitabine-treated MDS patients[130] with a median age of 70 years, IPSS score Int-1, n=45, Int-2, n=47 and high-risk, n=70. The response rate to decitabine was 49%. Thirty-one patients experienced progressive disease, most often already during the first two cycles. Thirty patients had stable disease. The response rate of the different IPSS subgroups was as follows: Int-1, 44%; Int-2, 51%; and high-risk, 51%. Patients above the age of 75 years had the same response rate as patients between 65 and 75 or below 65 years of age. A remarkable response in the platelet count was seen, with a significant increase in platelets in 42% of the patients already after one cycle of therapy. Of patients who received at least two cycles, 63% showed a significant platelet increment. The median response duration was 36 weeks (95% confidence interval, CI 31.9-40.1 weeks), with response durations for the different IPSS groups being; Int-1, 46 weeks; Int-2, 38 weeks; and high-risk, 36 weeks. The median survival was 15 months (95% CI 12.1-17.9 months) with a two-year survival of 34%. For the different IPSS subgroups these values were: Int-1, 23 months and 48%; Int-2, 15 months and 24 %; and high-risk 12 months and 27%. Age was not a significant prognostic factor for survival. Patients with a blast cell count >20 % and those with high-risk cytogenetic abnormalities had somewhat inferior survival data (median 11 months, 2-year survival 23%; and 15 months and 27%, respectively).[130]

The incidence and kinetics of cytogenetic responses in MDS patients following decitabine was also examined.[131] Of 115 successfully karyotyped patients, 61 (53%) had clonal chromosomal abnormalities prior to treatment. Major cytogenetic responses were observed in 19 patients (31% of those with abnormal cytogenetics, 17% of all patients by intention-to-treat) after a median of three courses (range, 2-6) until best cytogenetic response, i.e. approximately 18 weeks. The median duration of cytogenetic response was 7.5 months (range, 3-15). Grouping patients by IPSS (78), 3/5 cytogenetic responses (60%) were obtained in the IPSS *low-risk* group, 6/30 in the *intermediate risk* (20%) and 10/26 in the *high-risk* group (38%). Median survival in these cytogenetic subgroups was 30, 8 and 13 months, respectively. The relative risk of death in patients achieving a major cytogenetic response was 0.38 (95% CI 0.17-0.88), compared to that of patients in whom the cytogenetics were persistently abnormal (p=0.0213). In conclusion, repeated courses of low-dose decitabine induce cytogenetic remission in a substantial number of elderly MDS patients with pre-existing chromosomal abnormalities, with a significant survival improvement compared to that in patients in whom the cytogenetically abnormal clone persists.

Based on these results, low-dose decitabine appeared promising also in AML of the elderly. Four patients screened for 3 phase II studies of decitabine in high-risk MDS (PCH91-01, PCH95-11, PCH97-19) had already progressed to AML at the time of planned treatment start.[132] Due to their age and/or comorbidity (median age 77 years, range, 62-79) they were not eligible for intensive chemotherapy. The median performance status was 2 (range, 1-3). Comorbidity included coronary heart disease (2 patients), carotid artery stenosis, and heart failure. Two of the four patients had received previous treatment (one with low-dose DAC, the other with 6-mercaptopurine). The median WBC was 9.7/mL (range, 1.8-72), median bone marrow blasts 49% (range, 37-70). Anemia with hemoglobin <10.5 g/dL, and thrombocytopenia <50,000 platelets/μL was present in 4/4 and 2/4 patients, respectively. Three of the patients had cytogenetic abnormalities (sole 5q-, sole 7q-, complex karyotype). They received a median of 4 treatment courses (range, 2-8), resulting in an antileukemic effect (complete clearance of peripheral blood blasts and >50 % reduction of bone marrow blasts) in 2/4 patients. Two others achieved both a hematologic CR and karyotypic normalization lasting 5 and 11 months, respectively. Median survival from the start of DAC to death or last follow-up was 9 months (range, 5 to 20+). During that time, they spent a median of 66 nights in hospital (range, 57-101), which included all scheduled and unscheduled hospital admissions for any indication. Thus the median survival time spent at home was 76%.

We concluded that repeated courses of low-dose DAC treatment with this schedule in selected AML patients aged >60 years can result in hematologic CR and cytogenetic normalization. After brief hospitalization for DAC infusions, discharge and outpatient management was feasible, resulting in a substantial portion of the remaining lifetime not being spent in hospital. Reversal of *p15*^{INK4B} hypermethylation with generation of partly demethylated *p15* alleles, and induction of *p15* protein were observed by same group in bone marrow cells from MDS patients after the first course of decitabine treatment.[102] At this early treatment timepoint, persistence of an abnormal karyotype was noted in most cases, suggesting that demethylation occurred in clonal cells. Emergence of fully demethylated p15 alleles and reversion to normal karyotype with continued treatment are indicative of subsequent suppression of clonal growth.[102, 131]

Decitabine has recently been used at a comparable total dose (150 mg/m^2 total dose compared to 135 mg/m^2 in the European schedule) in the USA:[133] the recommended (minimal effective) dose of decitabine was 15 mg/m^2 in a 1-hour intravenous infusion daily for 10 days, thus resulting in a lower daily dose compared to that in the European schedule.[133] A total of 39 patients (mostly with pretreated AML) were enrolled in this study: 36 patients were evaluable, while 3 patients were excluded from the study (1 patient due to sepsis and death on treatment day 2 and 2 due to rapidly rising blood counts). Overall, the drug was well tolerated, with 1 death due to neutropenic sepsis, and 5 asymptomatic elevations in SGPT and/or bilirubin (4 grade 2, 1 grade 3). There were 7 complete remissions (19.4%) and 7 partial remissions (19.4%), with a response rate of 39% (95% CI 28% to 61%). Seven additional patients had significant reductions in peripheral and/or bone marrow blasts but never had normal hematopoiesis. Responses were observed in refractory/relapsed AML (10/30), MDS (3/4), and CML (2/2). In most patients who responded there was a gradual reduction of blasts over 2-4 weeks, and eventual recovery of normal hematopoiesis at 4-5 weeks, suggesting a not immediate cytotoxic mode of action also for this regimen. Response duration ranged from 2 to 10+ months. DNA methylation studies were also performed, showing *p15* demethylation already 5 days after treatment in 2 patients who subsequently achieved remission, which also indicates that low-dose decitabine induces remissions in part through demethylation rather than cytotoxicity.

Studies combining demethylating agents with other drugs

Decitabine has also been employed in combination with other anticancer agents, such as anthracyclines or, more recently, histone deacetylation inhibitors, e.g. phenylbutyrate. The initial phase II studies were performed in patients with relapsed and resistant leukemia, in combination with either amsacrine (120mg/twice daily for 3-6 days) or idarubicin (12 mg/m^2 for 3 days).[118]

The most important study was conducted by the *European Organization for Research and Treatment of Cancer (EORTC)*. 5-aza-2'-deoxycytidine, combined with either amsacrine or idarubicin,[134] was administered to patients with acute myeloid or lymphocytic leukemia in relapse. Sixty-three patients received 5-aza-2'-deoxycytidine 125 mg/m2 (decitabine) as a 6 h infusion every 12 h for 6 days (total dose 1500 mg/m^2), in combination with either amsacrine 120 mg/m^2 as a 1-h infusion on days 6 and 7 (n=30) or idarubicin 12 mg/m^2 as a 15-min infusion on days 5, 6 and 7 (n=33). Twenty-three patients (36.5%) achieved complete remission (8 of 30 patients treated with amsacrine and 15 of 33 treated with idarubicin). Complete remission was achieved by 51% of patients with more than a 1-year interval between initial diagnosis and start of therapy and in only 15.4% of patients with an interval shorter than 1 year. Patients with normal cytogenetics had a higher CR rate (61%) than those with abnormal cytogenetics (15.8%). With this high-dose decitabine schedule, digestive tract and hematologic toxicity was prolonged compared to that produced by standard induction schedules. The median disease-free survival was approximately 8 months, with only 20% of patients in remission for longer than 1 year. At this dose decitabine may be considered a good antileukemic agent, similar to cytarabine, but with considerable toxicity.

Preliminary results of a small phase II trial also combining an intensive schedule of decitabine with daunorubicin,[135] given as first-line induction therapy to patients with acute myeloid leukemia (except FAB M3), have been reported. Decitabine was given as a 4-h intravenous infusion at the dose of 90 mg/m^2 daily, days 1-5, while daunorubicin was administered at the dose of 50 mg/m^2 on days 1-3. A maximum of two courses were given with an interval of 4-6 weeks. Eight patients were enrolled and six of them were evaluable for toxicity and response. The main toxic effects were bone marrow suppression, mucositis, nausea and vomiting, and alopecia. All six patients achieved complete remis-

sion after one (5 cases) or two (1 case) courses.

Decitabine has recently been employed in patients with advanced MDS in combination with phenylbutyrate. Sodium phenylbutyrate (PB) is an aromatic fatty acid with cytostatic and differentiating activity against malignant myeloid cells (ID_{50}, 1–2 mM).[136,137] A number of mechanisms have been proposed for the antitumor effect of PB, including glutamine depletion[138] and inhibition of cholesterol synthesis.[139] The clinical activity of PB at low concentrations (0.25–0.5 mM) may be explained by its effect on histone acetylation. At these doses, like butyric acid, PB has an inhibitory activity on histone deacetylase, inducing histone H3 and H4 acetylation.[140] Like other histone deacetylase (HDAC) inhibitors,[141,142] PB synergizes *in vitro* with retinoids in the induction of differentiation and cell cycle arrest of myeloid leukemia cells. Furthermore PB, again like other HDAC inhibitors, synergizes with demethylating agents.[38,143-145] Patients with myelodysplasia (n=11) and AML (n=16) were treated with PB as a 7-day continuous infusion, repeated every 28 days, in a phase I dose-escalation study.[146] The maximum tolerated dose was 375 mg/kg/day; higher doses led to dose-limiting reversible neurocortical toxicity. At the maximum tolerated dose, PB was extremely well tolerated, with no significant toxicities. The median steady-state plasma concentration at this dose was 0.29-0.16 mM. Although no patients achieved complete or partial remission, four patients achieved hematologic improvement (of neutrophils in 3 patients, platelet transfusion-independence in 1 patient). Other patients developed transient increases in neutrophils or platelets and decrements in circulating blasts. Monitoring the percentage of clonal cells over the course of PB administration, using centromere fluorescence *in situ* hybridization, showed that hematopoiesis remained clonal. Hematologic response was often associated with increases in both colony-forming units-granulocyte-macrophage and leukemic colony-forming units. Administration of PB at higher doses was not feasible because of dose-limiting central nervous system toxicity, apparently due to accumulation of the metabolite PA (phase I studies of PA documented central nervous system toxicity as dose-limiting). Indications of hematologic activity were seen in all patients with MDS and AML receiving PB at all doses administered. Thus, at the plasma concentrations achieved, PB influences the expression of at least one surrogate gene involved in hematopoiesis. Recent demonstrations of the important role of histone acetylation in the regulation of gene expression[147] and the recruitment of histone deacetylase enzymes by several fusion genes involved in acute leukemias have led to the speculation that agents that inhibit histone deacetylase may be useful in the treatment of neoplasms. Inhibition of histone deacetylase may explain the changes in bone marrow CFU, hemograms, and F-cells seen in patients treated with PB, despite submillimolar plasma concentrations.

The outstanding toxicity profile of PB and the synergistic effect of histone deacetylation and demethylating agents in reactivating silenced genes[38] encouraged clinical studies on the combination of PB and demethylating agents in hematologic diseases characterized by *p15* silencing. Two clinical studies, NCI-sponsored, on 5-azacytidine (azacytidine) combined with PB, were presented at the ASH 2001 meeting. In the study of the Memorial Sloan Kettering Cancer Center,[144] the treatment scheme entailed subcutaneous injections of azacytidine for 7 consecutive days (75 mg/m^2/day), similar to the CALGB schedule, followed by 5 days of intravenous PB (200 mg/kg/day), repeated on a 21 to 28 day schedule, contingent on tolerability and response. At that time, 6 patients with myelodysplasia/secondary AML had received at least one cycle of therapy (range, 1-3). Reduction in bone marrow blast counts as well as increased myeloid maturation was observed in 4 patients; 1 patient with leukemia relapsed following bone marrow transplantation (BMT) had a complete elimination of bone marrow blasts after one cycle of therapy, and subsequently underwent a second allogeneic BMT. Peripheral blood and bone marrow samples were collected before azacytidine, on day 8 (at completion of azacytidine, and before beginning PB), and at the completion of PB. An increase in histone acetylation was consistently detected in peripheral blood and bone marrow samples collected after PB administration. Selected genes commonly silenced (eg. $p15^{INK4b}$ in myelogenous leukemia) were analyzed for methylation and expression. Changes in methylation of the $p15^{INK4b}$ (CDKN2b) promoter were assessed using real-time polymerase chain reaction.

Treatment was relatively well tolerated, with mild adverse reactions including fatigue, nausea, vomiting, and local tenderness at injection sites associated with 5-azacytidine and transient somnolence and drowsiness associated with PB. This ongoing study will evaluate the safety and potential antitumor efficacy of this combination, and its effects on gene methylation and histone deacetylation.

A second study combining azacytidine and PB was reported by the Johns Hopkins' Baltimore Group.[148]

Sequential administration of azacytidine and PB to re-express transcriptionally silenced genes was initated in patients with MDS and AML. The initial azacytidine dose was also 75 mg/m²/day subcutaneously, in this study given for 5 days, followed by PB at 375 mg/kg/day, i.v., continuous infusion, days 5-12 repeated every 28 days. Dose de-escalation to determine the minimal azacytidine dose associated with significant demethylation continues (current dose level 50 mg/m²/day). Eleven patients have been treated in a total of 39 courses. The combination was well tolerated, with 1/6 patients at the highest dose level developing dose limiting toxicity (fatigue). No unexpected clinical toxicities have been observed to date. Two patients had significant hematopoietic improvement. The primary laboratory end-point is inhibition of methylation. Baseline methylation activity was highly variable. Of two patients with high baseline activity, one had significant inhibition (85% of baseline levels) following treatment with 5-azacytidine (50 mg/m²/day). Two of 5 patients with lower baseline levels of activity also showed inhibition (20 and 30%) following treatment with 75 mg/m²/day. Histone acetylation was increased over baseline in 4/11 patients investigated. In addition, 2 patients had significant detectable acetylation which persisted during the PB infusion. Increases in acetylation were detected within 4 hours of initiation of PB infusion and persisted throughout the infusion. In 9 patients, sequential measurements of $p15^{INK4B}$ promoter methylation by a newly developed PCR-based assay were performed. All had measurable methylation of the *p15* promoter exceeding 10% of available CpG sites (normal < 2%). *p15* methylation density was higher in patients with AML or RAEB-t compared to in patients with lower-grade MDS. In 3 patients during 5- azacytidine/PB treatment, *p15* methylation levels decreased to 19%, 45%, and 56%. *p15* methylation increased in 1 patient (in association with disease progression) and was stable in 5 patients. Baseline methylation density did not predict the extent of demethylation in response to 5 azacytidine/PB.

Both studies demonstrate that the sequential administration of a *first generation* demethylating agent and HDAC inhibitors is feasible, and give preliminary evidence of an effect on the methylated targeted gene promoter, as also described with decitabine.[102]

Conclusions

Although supportive care remains the standard therapy for low-risk MDS, a number of treatment approaches aiming at improving cytopenias in transfusion-dependent patients are currently under investigation. Among others, immunosuppressive, anticytokine, and antiangiogenic therapy have been proposed. Transcriptional silencing of tumor suppressor genes occurs in cancer cells by DNA methylation and by histone deacetylation (HDAC). Recently, novel agents that target these mechanisms have been developed. At present, the demethylating agents azacytidine and decitabine are the most promising drugs for the treatment of elderly patients with high-risk MDS or for young patients without a compatible donor. Combinations with histone deacetylation inhibitors, such as sodium phenyl-butyrate, could further increase the efficacy of these drugs.

Contributions and Acknowledgments

GL: responsible for all Tables and Figures 1 and 2: MTV: adaptation of Figures 3 and 5; Figure 4: courtesy of Dr. Clara Nervi.

Funding

This work was supported by grants from M.U.R.S.T (Ministero dell'Università e della Ricerca Scientifica e Tecnologica), A.I.R.C. (Associazione Italiana per la Ricerca sul Cancro), Wilhelm-Sander-Stiftung (grant 99.032.1) and the Deutsche Forschungsgemeinschaft (Lu429/5-1).

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PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two inhouse referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken by Professor Cazzola. Manuscript received October 11, 2002; accepted October 15, 2002.

Mario Cazzola, Editor-in-Chief (Pavia, Italy)